Newborn resuscitation

An experimental study of inflammatory and hemodynamic responses in newborn pigs

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To Ida & Anne

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Contents

Publications

This thesis is based on three publications referred to by Roman numbers throughout the text:

I **Dannevig I**, Solevåg AL, Wyckoff M, Saugstad OD, Nakstad B
Delayed onset of cardiac compressions in cardiopulmonary resuscitation of newborn pigs with asphyctic cardiac arrest.
Neonatology 2011; 99(2): 153-62.

II **Dannevig I,** Solevåg AL, Sonerud T, Saugstad OD, Nakstad B
Brain inflammation induced by severe asphyxia in pigs and the impact of alternative resuscitation strategies on the newborn central nervous system.
Pediatric Research 2013 Feb; 73(2): 163-70.

III **Dannevig I,** Solevåg AL, Saugstad OD, Nakstad B
Lung injury in asphyxiated newborn pigs resuscitated from cardiac arrest - the impact of supplementary oxygen and longer ventilation intervals before initiation of chest compressions at different compression-to-ventilation ratios.
Open Respir Med J. 2012; 6: 89-96.

Abbreviations

American Academy of Pediatrics (AAP)
Brain Natriuretic Peptide (BNP)
Bronchoalveolar lavage fluid (BALF)
Bronchopulmonary dysplasia (BPD)
Cardiopulmonary resuscitation (CPR)
Central nervous system (CNS)
Cerebrospinal fluid (CSF)
Compression:Ventilation ratio (C:V)
Electrocardiogram (ECG)
Electroencephalogram (EEG)
Enzyme-linked Immunosorbent Assay (ELISA)
European Resuscitation Council (ERC)
Functional Residual Capacity (FRC)
Heart rate (HR)
Hypoxic-ischemic encephalopathy (HIE)
Initial fraction of oxygen ($iFiO_2$)
Interleukin (IL)
Interquartile range (IQR)
Mean arterial blood pressure (MABP)
Metalloproteinase (MMP)
Near infrared spectroscopy (NIRS)
Partial arterial Pressure of Oxygen (PaO_2)
Peak Inspiratory Pressure (PIP)
Postive end-expiratory pressure (PEEP)
Positive pressure ventilation (PPV)
Regional cerebral oxygen saturation ($rScO_2$)
Relative Quanitification (RQ)
Respiratory distress syndrome (RDS)
Return of spontaneous circulation (ROSC)
Soluble Intercellular Adhesion Molecule (sICAM)
Sustained inflations (SI)
The European Resuscitation Council (ERC)
The International Liaison Committee on Resuscitation (ILCOR)
Tumor necrosis factor (TNF)

What is this thesis about?

Most newborns are born vigorous and do not require neonatal resuscitation (1, 2). In almost all infants, the changes from intrauterine to extrauterine life are successfully completed at delivery without requiring any special assistance. About 10% of newborns require some type of resuscitative assistance at birth (1, 2). Resuscitation of the newborn requires rapid intervention to avoid circulatory insufficiency and cerebral hypoperfusion. The vast majority will require just assisted lung aeration, but about 1% will require bag-and-mask and/or endotracheal positive-pressure ventilation, administration of oxygen and, more rarely, chest compressions and use of drugs such as adrenaline (1-3).

Guidelines for neonatal resuscitation have until relatively recently been based on expert opinion, case series and tradition. The developer of the Apgar score, Virgina Apgar, said in the early 1950s that “Resuscitation of infants at birth has been the subject of many articles. Seldom have there been such imaginative ideas, such enthusiasms, and dislikes, and such unscientific observations and study about one clinical picture. There are outstanding exceptions to these statements, but the poor quality and lack of precise data of the majority of papers concerned with infant resuscitation are interesting” (4).

Over the past two decades, evidence-based guidelines have finally been developed (3). The quality of evidence has improved and recommendations have been derived from evaluation of the available scientific literature (3, 5). However, there are still several aspects of the algorithm for newborn resuscitation that are in need of further research. Finer and Rich reviewed the topic in 2004 and noted that neonatal resuscitation still was a “poorly studied intervention” despite its importance (6). They described a lack of evidence to support the guidelines.

My overall aim with this thesis was to explore if alternative resuscitation protocols would be better than the accepted algorithm. Would longer ventilation intervals before initiation of chest compressions result in shorter time to return of spontaneous circulation (ROSC) and a lower inflammatory response in brain and lung tissue? We also challenged the guidelines on the compression to ventilation ratio (C:V) of 3:1 and the impact of supplementary oxygen supplied to newborns at cardiac arrest. We chose the newborn pig model to investigate these aspects of newborn resuscitation because it resembles humans in anatomy and physiology of the cardiovascular system (7-9).

Introduction

Resuscitation of the newborn and ILCOR guidelines

Newborn infants that are born at term and are breathing or crying and have good muscular tone, should only be dried and kept warm (3). These steps can be provided with the baby lying on the mother`s chest.
The newborns in need of advanced resuscitation should be assessed to determine their need for one or more of the following actions (3):

1: Initial steps in stabilization (dry and provide warmth, position, assess the airway, stimulate to breathe)

2: Ventilation

3: Chest compressions

4: Medications

In the most demanding situations, resuscitation requires rapid intervention to avoid circulatory insufficiency and cerebral hypoperfusion. Some will require bag-mask ventilation only, but about 1% will require bag-and-mask and/or endotracheal positive pressure ventilation (PPV) and more rarely, 0.12% of newborns need chest compressions and use of drugs such as adrenaline (3).

Among several established newborn resuscitation guidelines, the most referenced worldwide are those of the International Liaison Committee on Resuscitation (ILCOR). In 1992, the ILCOR was established, aiming to provide a forum for the main resuscitation organizations in the world, including the American Heart Association (AHA), American Academy of Pediatrics (AAP), and the European Resuscitation Council (ERC), to establish international guidelines (10). The creation of ILCOR established a unique opportunity for international collaboration in the development of guidelines and training programs on resuscitation over the past twenty years. One of the main objectives of ILCOR is to produce practice statements that reflect international consensus on resuscitation-specific issues for patients of any age. The international resuscitation guidelines are revised every five years after an extensive evidence evaluation (10). The flowchart of the valid newborn guidelines when we started the experimental series underlying this thesis is shown in figure 1 (11), as compared to the present ILCOR guidelines in figure 2 (3).

In the guidelines from 2010, ILCOR now recommends to begin with air rather than air enriched with oxygen, and specifically not 100% oxygen in term and near term infants receiving ventilation at birth (3). The evaluation and decision to initiate resuscitation of the newborn should primarily be based on heart rate and respiration, and not skin color (3, 12). Initial assisting ventilation, if needed, should be performed for 30-60 seconds and you should not suction the baby just because the amniotic fluid is meconium stained (3, 12). The guidelines released in 2010 are based on the most recent International Consensus on Cardiopulmonary Resuscitation (CPR) Science with Treatment Recommendations, which incorporated the results of systematic reviews of a wide range of topics relating to CPR, including the resuscitation of the newly born in the delivery room (3, 12).

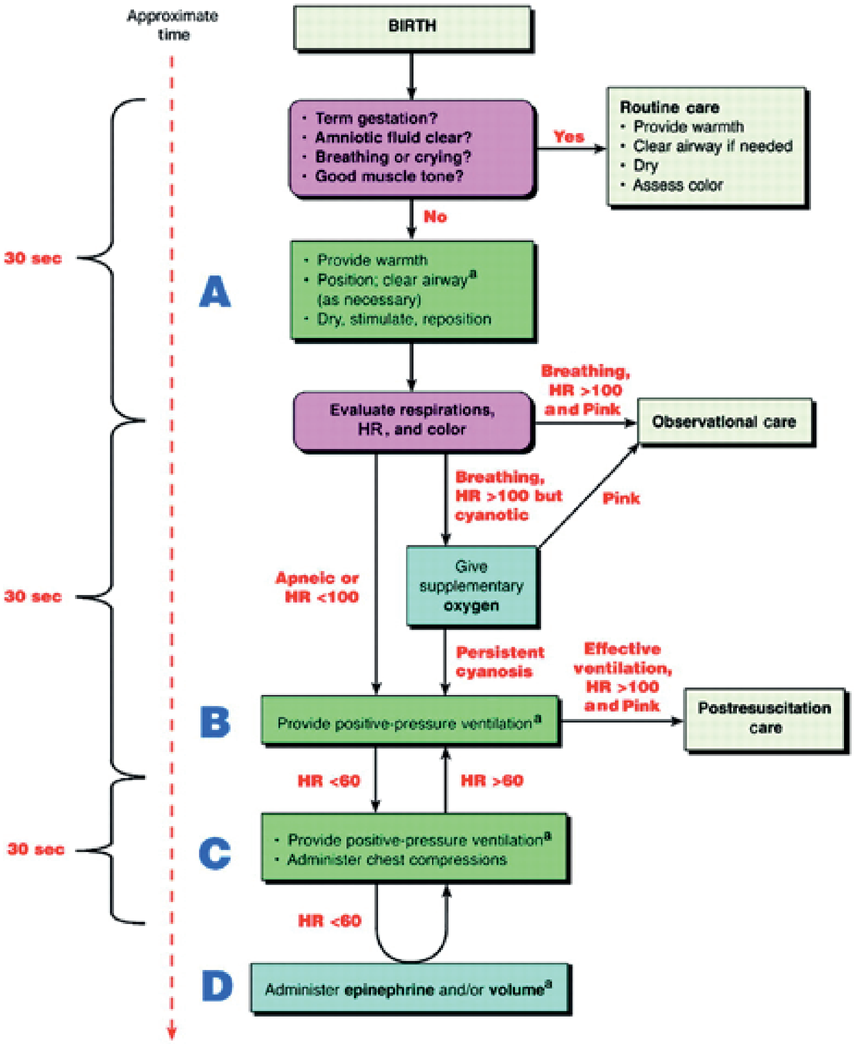

Figure 1. Algorithm for newborn resuscitation at the time when our studies were initiated (11). ©2006 by American Academy of Pediatrics. Reprinted with permission.

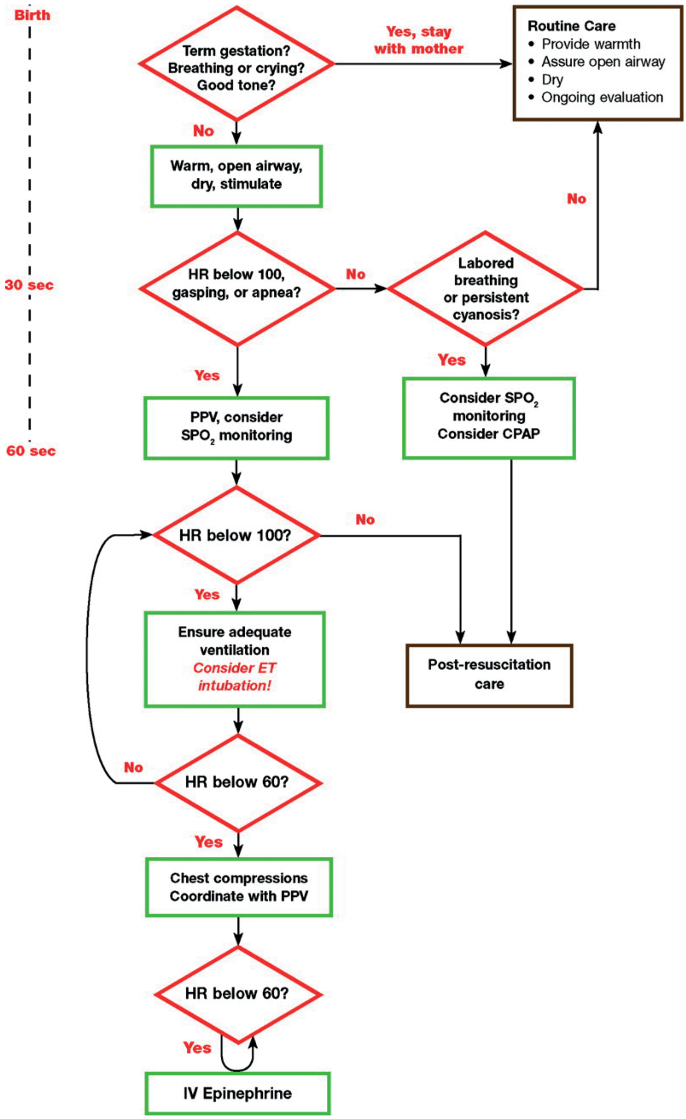

Figure 2. The current 2010 ILCOR algorithm for newborn resuscitation (3) with permission from Elsevier.

Some of the new changes in the 2010 ILCOR guidelines (3, 12)

- For babies born at term, resuscitation should begin with air rather than 100% oxygen.

- Administration of supplementary oxygen should be regulated by blending oxygen and air, and the oxygen concentration delivered should be guided by oximetry.

- Progression to the next step following the initial evaluation is now defined by simultaneous assessment of 2 vital characteristics, namely heart rate and respirations. Oximetry should be used for evaluation of oxygenation because assessment of color is unreliable.

- The chest compression-to-ventilation ratio should remain at 3:1 for neonates unless the arrest is known to be of cardiac etiology, in which case a higher ratio should be considered.

- The available evidence does not support or refute the routine endotracheal suctioning of infants born through meconium-stained amniotic fluid, even when the newborn is depressed.

- Cord clamping should be delayed for at least 1 minute in babies who do not require resuscitation. Evidence is insufficient to recommend a time for clamping in those who require resuscitation.

- Therapeutic hypothermia should be considered for infants born at term or near-term with evolving moderate to severe hypoxic-ischemic encephalopathy, with protocol and follow-up coordinated through a regional perinatal system.

Resuscitation of the newborn and ventilation strategies

The transition from intrauterine to extrauterine life requires the lungs to change from being fluid-filled to air-filled. Approximately 10% of newborns do not achieve this transition without assistance to begin breathing (2). The 2010 ILCOR guidelines advocate the establishment of effective ventilation as the primary objective in the management of the apneic or bradycardic newborn infant in the delivery room (3, 12). Considering the frequency with which neonatal resuscitation is performed, and the variety of devices available for resuscitating newly born infants, there have been relatively few prospective, randomized, clinical studies in newborns on which to base recommendations for practice.

However, the effectiveness of PPV alone in the resuscitation of asphyxiated term newborns was established already in the 1960s through a series of animal experiments (13-17). These studies provided important descriptions of the cardiorespiratory responses to severe asphyxia in newborn mammals of several species including rabbits, monkeys and lambs. Chest compressions were initiated if the heart rate did not increase within 30-45 seconds of ventilation. These studies show that PPV alone is effective for resuscitation of apneic, bradycardic newborn animals, provided that mean arterial blood pressure is above a critical value. The first sign of succsessful resuscitation was a rapid increase in heart rate (figure 3).

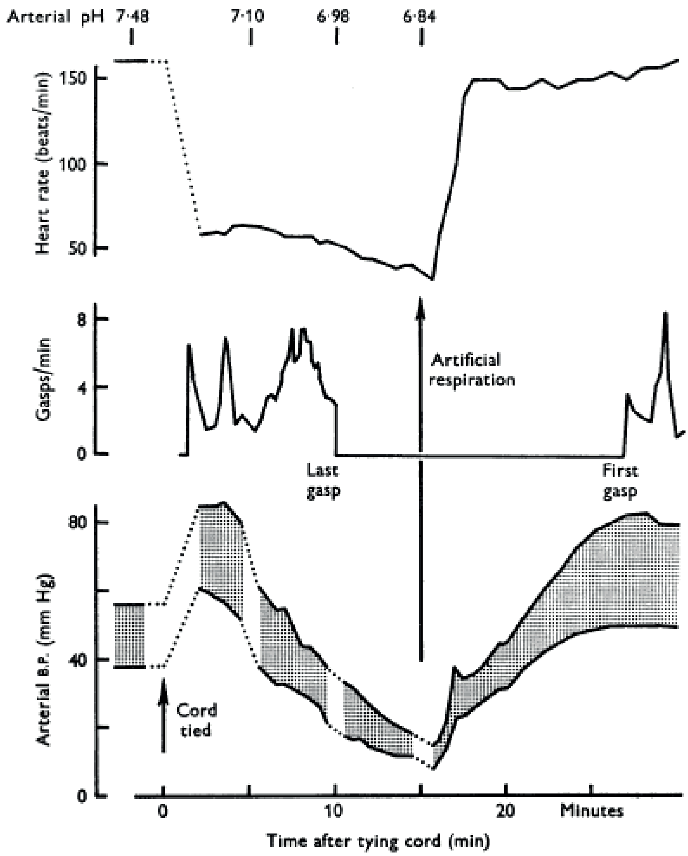

Figure 3. Response to asphyxia and resuscitation (from ref. (16)). Reprinted with permission.

The level to which arterial blood pressure decreases before chest compressions are necessary, differs between species. Mean arterial blood pressure (MABP) is 45-60 mmHg in newborn monkeys and lambs, and the need for chest compressions is associated with a MABP < 15 mmHg (15, 16). The MABP in newborn rabbits is approximately 30 mmHg and chest compressions do not seem to be necessary until the MABP has decreased to < 5-8 mmHg (13).

Most apneic newborn infants respond well to aeration of the lungs. The oxygen saturation of the fetus just prior to delivery is about 50%, and the average healthy newborn requires about 5 min to increase the oxygen saturation to above 90%, with many healthy infants requiring longer periods (18). Preterm infants have amplified difficulties with adapting to air breathing and often need assistance for the transition (19).

The most important and fastest indicator of initial lung inflation is an improvement in the baby`s heart rate. If the heart rate does not increase quickly, ventilation might not be effective or adequate (20). Leak and obstruction with mask ventilation during simulated neonatal resuscitation show that this may be a major problem (21, 22) as also shown in delivery room studies where mask leak and airway obstruction are common problems during PPV (23).

In 1995, Perlman and Risser performed an observational study and found that chest compressions or adrenaline (epinephrine) were administered in 0.12% of delivery room resuscitations (1). In approximately two-thirds of the infants who received chest compressions or adrenaline, ineffective or improper initial ventilatory support was the presumed mechanism for continued neonatal depression. The findings of that study confirm the primary importance of proper, effective initial ventilatory support in resuscitation of asphyxiated newborns.

Evaluation of lung inflation is difficult due to provider anxiety, operator position, crowded bed space, infant size, and the use of a plastic wrap for the premature newborns. A study assessing the adequacy of resuscitation at birth, demonstrated that even experienced neonatal staff find it difficult to determine whether chest wall expansion is adequate (24). In addition, CPR competency and mastery are transient skills. Poor skill performance of diverse providers 3 to 6 months after CPR training has been recognized (25-27). Some studies have even shown a fast decline in resuscitation skills after only 2 to 3 weeks (28). Repetitive reinforcement and refreshment of skills is essential and necessary in order to impact provider competence and newborn survival (29-32).

In newborn resuscitation, assisted ventilation rates of 40-60 breaths per minute are commonly used, but evidence supporting this is lacking.
In a manikin study examining ventilation and chest compression dynamics, our research group showed that ventilation rates of either 40 or 60 per minute both gave adequate ventilation tidal and minute volumes, with a correlation between ventilation rate and minute volumes despite a negative correlation between ventilation rate and tidal volumes (22).

Progression in the resuscitation algorithm should first ensure that appropriate ventilation volumes are provided. The initial peak inflating pressures (PIP) should be sufficient to achieve an increase in heart rate and/or chest wall movement. An initial PIP of 20 cm H_2O may be effective, but 30 to 40 cm H_2O may sometimes be required (33).

Mask ventilation during neonatal resuscitation is often hampered by large leaks at the face mask (34, 35), and when efforts are taken to minimise leaks, moderate or total airway obstruction may occur. Training in mask ventilation should be included in training programs to minimise leakage and to prevent airway obstruction.

An ongoing in-delivery-room clinical and in-between refresher trial (INSPIRE-D) run at Akershus University Hospital, Oslo University Hospital and Childrens Hospital of Philadelphia, USA, addresses the mask-bag ventilation techniques of midwifes and pediatricians. This will give important clinical information and pave the way for improved ventilation techniques among the staff. Previous manikin studies have shown that training in mask ventilation have reduced mask leaks, but they also remind us that we should also focus on preventing airway obstruction (21). An airtight seal between the mask and the face is important for successful ventilation, but too much pressure applied to the mask to prevent leak may on the other hand lead to obstruction of the mouth and nose (36).

The 2010 ILCOR guidelines presented a simplified algorithm that highlighted the central role of respiratory support during resuscitation of the newborn (3). According to the same guidelines, establishing initial lung inflation in apneic newborn infants, can be accomplished with either shorter or longer inspiratory times (12). The AHA recommends that during neonatal resuscitation, assisted ventilation should be delivered at a rate of 40 to 60 breaths per minute but does not explicitly suggest using longer inflation times for the initial inflations (37). In contrast, the revised European guidelines recommend that one should maintain the inflation pressure for 2-3 s for the first five inflations as this will help to expand the lungs (5). Subsequently, babies should be ventilated at a rate of about 30 breaths per minute allowing approximately 1 s for each inflation (5).

Vento et al (38) suggest that bringing intensive care technology for measuring inspiratory and expiratory flow and ventilation volumes to the delivery room may improve neonatal outcomes. Volume-targeted ventilation is widely practised and could reduce rates of pneumothorax, bronchopulmonary dysplasia and severe intraventricular hemorrhage in the neonatal intensive care unit (39).

As per today we do not know the optimal ventilation strategy immediately after birth when the lungs are liquid-filled. In term-asphyxiated infants, there is a lack of evidence to support any specific ventilation strategy for the initial inflations. Several recent studies have, however, shown that one sustained inflation (SI) of 2-5 s could improve functional residual capacity (FRC) and a more rapid circulatory recovery compared with an approach not using a SI (40-43). Initiating respiratory support with a SI, might improve FRC development by allowing an appropriate time-constant for the air/liquid interface to move into distal airways. Without having achieved a FRC in the lungs, blood will not become oxygenated in prolonged resuscitation. The SI technique may give a quicker formation of FRC than other ventilation techniques and can affect clinical outcome (42). This thesis does not include experiments with SI, but this issue is and will be a focus of future studies in our research group.

Resuscitation of the newborn and cardiac compressions

The infrequent use of cardiac compressions for newborns in the delivery room has led to investigations to find the most effective neonatal cardiac compression methodologies (44). Cardiac compressions are estimated to occur in approximately 1 in 1000 term deliveries, with a higher frequency in preterm infants (1). Cardiac compressions achieve only a fraction of native perfusion even under optimal conditions, so optimising compressions could be critical in improving outcomes (45).

Cardiac compressions are indicated when the heart rate is less than 60 beats per minute despite adequate ventilation (3, 12). In contrast to the resuscitation guidelines for children and adults, guidelines for neonatal resuscitation recommend 90 cardiac compressions synchronized with 30 manual inflations (3:1) per minute (3, 12). In animal models of asphyxia at cardiac arrest, pigs resuscitated with a combination of cardiac compressions and ventilations had better outcomes than those resuscitated with ventilations or compressions alone (46, 47). If the arrest is clearly due to a cardiac etiology, a higher compression:ventilation (C:V) ratio, e.g. 15:2 may be considered (3, 12). However, because ventilation is critical to reversal of newborn asphyxial arrest, any higher ratio that decreases minute ventilation should be introduced with caution (3).

The two main goals of providing perfusion via cardiac compressions are to reperfuse the heart and the brain. If the myocardium is not adequately perfused with too low diastolic pressures as a surrogate for coronary perfusion pressure (CPP), resuscitation efforts can be unsuccessful (44). Adequate coronary perfusion is necessary to deliver oxygen to the myocardium so that energy (ATP) can be regenerated in hopes that effective myocardial function and return of spontaneous circulation (ROSC) can be reestablished (44).

There are two possible methods that can be used during cardiac compressions: the two thumb-encircling hands, also used in the experimental studies underlying this thesis, and the two-finger method. The two thumb-circling hands method is recommended because it is less tiring and allows for better cardiac compression depth control (48). Randomised studies in pigs (49, 50) and manikins (48, 51, 52) as well as small case series (53), support the current practice of favouring the two thumb-encircling hands technique of cardiac compressions when compared with the two-finger technique. Compressions should be delivered over the lower third of the sternum rather than the mid-sternum (54, 55) and the depth of the cardiac compressions should be one third of the external anterior-posterior diameter of the chest rather than deeper cardiac compressions (56). It is also of paramount importance to release the pressure on the chest between every chest compression so that circulating blood can refill the heart and then in the next caridac compression be pushed out of the heart again like in the systole.

Newborns that require prolonged cardiac compressions with no signs of life beyond 10 minutes are at risk for exceptionally poor outcomes, with up to 83% mortality and 77% severe disability noted in survivors (57). Optimization of the hemodynamics of neonatal cardiac compressions during CPR remains critical. The current recommendation for a 3:1 C:V ratio is based on the physiological plausibility that even healthy newborns have higher respiratory rates compared to adults or older children; hence higher ventilation rates during CPR in newborns are required. Optimized cardiac compressions can only achieve approximately 30% of normal perfusion (45, 58). However, du to preferential perfusion of the heart and brain during cardiac compressions, myocardial and cerebral blood flow of greater than 50% of normal may be achieved (59-61). This is of significance for restoration of oxygen delivery to these vital organs. Still, data on morbidity and mortality for both preterm (62, 63) and term infants (2, 57) who receive cardiac compressions in the delivery room, suggest a need for optimization of neonatal cardiac compressions.

Resuscitation of the newborn and the use of oxygen

Previous international guidelines uncritically recommended use of 100% oxygen for newborn resuscitation. During years of experimental work by Saugstad's group and others, as well as clinical studies, doubts about this oxygen therapy practice were raised (64). Already in 1992, a study in newborn pigs showed that heart rate and metabolic changes in asphyxiated newborn pigs returned to normal just as quickly with 21% oxygen as with 100% oxygen during assisted ventilation (65).

The past decades, we have seen remarkable changes in attitudes towards the use of oxygen in delivery room resuscitation. Meta-analyses have shown a decrease in mortality for the groups resuscitated with air (66-69).

Davis et al did in 2004 a systematic review and meta-analysis on resuscitation of newborn infants with 100% oxygen or air and concluded that air should be used initially for term and near-term infants, with oxygen as backup if initial resuscitation fails (68). The effect of intermediate concentrations of oxygen at resuscitation needs to be further investigated.

A meta-analysis and systematic review of seven randomized and quasi-randomised controlled trials by Rabi et al. (66) assessed the effects of room air resuscitation versus 100% oxygen resuscitation on mortality at 1 week and 1 month in asphyxiated newborn infants. Compared to the 100% oxygen resuscitation group, neonates in the room air resuscitation group had a lower mortality both in the first week of life (odds ratio 0.70, 95% CI 0.50, 0.98) and at 1 month and beyond (odds ratio 0.63, 95% CI 0.42, 0.94). This meta-analysis supports the hypothesis that room air is superior to 100% oxygen as the initial choice for resuscitating clinically depressed newborns as it may result in a lower mortality rate.

It has been a recognized that 100% oxygen can be harmful to both preterm and term infants (70). Several newborn animal models of asphyxia have found that high concentrations of oxygen during resuscitation can be potential harmful at a cellular level (71, 72). The oxygen debate has now shifted to questions of how to best titrate oxygen delivery to an infant. The infant`s requirements and the concept of targeting ”normoxia” throughout neonatal resuscitation has been introduced (73).

Guidelines for resuscitation of neonates had last been published in 2005 (74) when we initiated our studies. Specifically the ILCOR guidelines in 2005 did not specify the concentration of oxygen to be used, but recommended that

supplementary oxygen should be considered for infants with persistent central cyanosis (74).
Since 2010, the European Resuscitation Council guidelines advise to start resuscitation in term infants with air rather than 100% oxygen and to follow oxygen saturation (SpO_2) targets for the first 10 min after birth (5, 75). These targets are based on observational studies by Dawson et al (76). They measured SpO_2 in the first 10 min after birth in 468 infants who received no medical intervention in the delivery room (76). These data were used to develop reference ranges for SpO_2 in the first 10 min after birth for preterm and term infants. The authors suggest that median oxygen saturations rise steadily from around 60% at 1 min of age to above 90% by 10 min. At present, this reference range represents our ”best guess” at optimal oxygen saturation targets. What saturation levels one should aim for remains to be established.

Clinical studies of newborns in need of resuscitation have indicated that those receiving PPV with air had a higher Apgar score at 5 min, higher heart rate at 90 s of age and took their first breath 30 s earlier than those who received 100 % oxygen (69). Pure oxygen seems to trigger a long-term increase in oxidativ stress and more injury to the myocardium and kidney (77). There is also evidence in newborn animal models of asphyxia that exposure to high concentrations of oxygen at resuscitation does not confer any clinical advantage and is potentially harmful at the cellular level (71, 72).

In 2001, Solas et al published a study on newborn pigs resuscitated with 21% or 100% oxygen (78). Cerebral hypoxia-ischemia was achieved by normoventilation with 8% oxygen and temporary occlusion of both common carotid arteries. They studied effects on extracellular levels of excitatory amino acids and microcirculation, and found a less favorable outcome in the group resuscitated with room air. This is in contrast to previous findings in the global hypoxemia pig model (65, 79). Following ischemia, perfusion abnormalities and microcirculatory disturbances play an important role in reperfusion injury. If the ischemic insult is severe, the no-reflow phenomenon may occur with failure of reperfusion in various areas of the brain (80). Under such conditions, an increase in arterial oxygen content may be of benefit to borderline perfused areas with better restoration of microcirculation as a result (81). Solas later repeated the study and added hypercapnia during the insult to mimic the clinical situation of asphyxia and found that CO_2 served to protect the brain, the differences in MABP and microcirculation was less marked (82).

Markus et al found an increase in cerebral gene expression of Interleukin (IL)-1β after ventilation with 100% oxygen compared to room air after fetal asphyxia in a newborn sheep model (83). In hypoxic newborn mice and pigs, oxygen exposure delays DNA repair after oxidative damage (72, 84). Despite this evidence that 100% oxygen exposure of asphyxiated animals is harmful, international guidelines still recommend 100% oxygen to be used whenever cardiac compressions is needed. This recommendation is based on the lack of evidence about the effectiveness of lower oxygen fractions in infants with the most severe compromise needing cardiac compressions.

The demonstration that 21% oxygen is as effective as 100% oxygen in combination with cardiac compressions in newly born pigs with brief asystole, was first reported by our research group (85). Linner et al made the same conclusion in asphyxiated pigs with severe bradycardia and hypotension needing cardiac compressions. These results should be interpreted in relation to the important finding that use of air during resuscitation in human newborns is associated with lower mortality. Systematic reviews and meta-analyses show that neonatal mortality can be reduced by 30% in newborns resuscitated with room air (68, 69).

Perinatal asphyxia and hypoxic-ischemic encephalopathy

Hypoxic-ischemic encephalopathy (HIE) is a major cause of neurologic disabilities in term neonates despite the recent widespread use of hypothermia therapy. The incidence of HIE ranges from 1 to 8 per 1000 live births in developed countries and is as high as 26 per 1000 live births in underdeveloped countries (86). The injury evolves over the course of days and possibly weeks.

Adequate cerebral blood flow delivers oxygen and glucose to the fetal brain. This blood flow helps the fetal brain maintain homeostasis and meet cellular energy demands. The hypoxia eventually leads to a decrease in fetal cardiac output, which reduces cerebral blood flow and sets in motion a temporal sequence of injury, which clinicians have divided into distinct phases. In the acute phase, the decreased cerebral blood flow reduces the delivery of oxygen and glucose to the brain, which leads to anaerobic metabolism.
Depending on the timing of injury and the degree of medical intervention, a partial recovery occurs during the 30 to 60 minutes after the acute insult or the primary phase of injury. This partial recovery ushers in a latent phase of injury (87). The latent phase may last from 1 to 6 hours and is characterized by recovery of oxidative metabolism, inflammation, and continuation of the activated apoptotic cascades (88). A secondary deterioration follows the latent phase in neonates with moderate to severe injury. The secondary phase of injury occurs within approximately 6 to 15 hours after the injury. Cytotoxic edema, excitotoxicity, and secondary energy failure with nearly complete failure of mitochondrial activity characterize this secondary phase, which leads to cell death and clinical deterioration in neonates with moderate to severe injury (87). Seizures typically occur in the secondary phase (89). A tertiary phase occurs during the months after the acute insult and involves late cell death, remodeling of the injured brain, and astrogliosis (90).

Perinatal asphyxia is a result of compromised gas exchange in the placenta or in the lungs, and features low oxygen delivery to tissues. Characteristic of the condition include low oxygen saturation and hypoxemia (low partial pressure of oxygen (pO_2) in the blood), hypercapnia (increased partial pressure of carbon dioxide (pCO_2) in the blood) and a combined respiratory and metabolic acidosis. Severe hypoxia (oxygen deficiency causing impaired tissue metabolism) results in anaerobic glycolysis and lactic acid production in peripheral tissues and the brain, and causes the metabolic component of the acidosis. Ischemia (reduced blood supply to organs and tissue) is both a cause and a result of hypoxia, as hypoxia and acidosis depress myocardial function with resulting hypotension and hypoperfusion of the heart and further compromise in the delivery of oxygen and removal of carbon dioxide and lactate (91). Perinatal asphyxia may occur antepartum (20%), intrapartum (35%), postnatally (10%) or as a combination (92). An cord umbilical artery pH < 7.0 and/or BE < -16 mmol/l has been found to correlate with an increasing risk of neurological deficit (93).

Neonatal HIE is an acute, nonstatic condition caused by brain hypoxia and ischemia during or closely associated with labor. It does not refer to any particular cause or timing, it includes any type of brain injury, insult or condition that results in central nervous system dysfunction. This includes infection, focal infarction, intracranial haemorrhage, hypoxia-ischaemia, metabolic diseases and hypoglycaemia (94).

Neonates with suspected HIE are classified according to the Sarnat staging system (95), which evaluates the level of consciousness, muscle tone, tendon reflexes, complex reflexes, and autonomic function. The Sarnat stage classifies neonatal HIE into the following 3 categories: stage I (mild), stage II (moderate), and stage III (severe). Entry criteria for therapeutic hypothermia include a modified version of the Sarnat staging system, table 1.

Modified Sarnat and Sarnat score		
HIE Grade I (mild)	HIE Grade II (moderate)	HIE Grade III (severe)
Irritability “Hyper alert” Mild hypotonia Poor sucking	Lethargic Seizures Marked abnormalities of tone Requires tube feeding	Comatose Prolonged seizures Severe hypotonia Failure to maintain spontaneous respiration

Table 1. Clinical grading of HIE, first published by Sarnat (95) and further modified by Levene (96).

In the brain, hypoxia-ischemia initially causes energy failure and loss of mitochondrial function. This is accompanied by membrane depolarization, brain edema, an increase of neurotransmitter release and inhibition of uptake, and an increase of intracellular calcium that causes additional pathologic cascades (97). These include oxidative stress, with the production of reactive oxygen species and interaction with the nitric oxide pathway to produce reactive nitrogen species (98). Both gray and white matter injury occur in term neonates with HIE. In term infants, the cortex, basal gangliae and thalami are particularly vulnerable to hypoxia-ischemia, while isolated white matter injury is more rare (99, 100).

There are no established biomarkers for determining the extent of neonatal brain injury or predicting outcome in infants with hypoxic-ischemic encephalopathy.

In some studies, serum IL-1β, serum IL-6, and cerebrospinal fluid IL-1β (all measured before age 96 hours) were possible markers of abnormal outcomes at age ≥12 months in survivors (101). In one study of term newborns with neonatal encephalopathy that were treated with hypothermia, levels of serum S100 correlated with neurologic outcomes (102). Further studies are needed to determine if any of these markers truly could be useful for the early assessment of infants with neonatal encephalopathy and hopefully indicate if therapy should be started.

Hypothermia is currently the only effective neuroprotective therapy available for treatment of HIE. It is relatively easy to administer, although several clinical parameters need to be recorded, such as hemodynamics, electroencephalogram (EEG) and temperature. The procedure however seems to be safe. According to a meta-analysis of seven randomized controlled trials of therapeutic hypothermia involving 1214 newborns with moderate to severe HIE, treatment with hypothermia improves survival and neurological outcome at 18 months after perinatal asphyxia and/or HIE (103).

A combination of treatment methods targeting different mechanisms of injury may be necessary for achieving the best possible outcome after perinatal asphyxia (104). Recent clinical research now focuses on the additive effects of hypothermia in combination with other neuroprotective treatment options (105). Hypothermia changes the mechanisms of injury after perinatal asphyxia, as well as the pharmacokinetics and pharmacodynamics of drugs. It is important and necessary to find the optimal time window for treatment, combinations of treatments, drug doses and any side effects both in vitro and in animals before clinical trials take place.

Resuscitation of the newborn and brain inflammation

Neuronal injury related to inflammation can induce a cascade of immune responses that are involved in the pathogenesis of early brain injury (106-108). Neuroinflammation is linked to the aggravation of early brain injury following hypoxia-ischemia and alone serves as a cause for brain injury (106). At the cellular level, reduction of cerebral blood flow and oxygen delivery can initiate a cascade of biochemical events leading to acidosis and cell death. Neurons and oligodendroglia continues to die over hours, days, or possibly weeks and months (109, 110).

The mechanisms involved in inflammation-induced early brain injury in the newborn are not clearly understood, and the origin of inflammatory signaling substances, including cytokines may vary. Recent research has shown that inflammatory responses can contribute to inflammatory cerebral white matter damage (75, 109, 111). The inflammatory signal substances may be produced systemically and distributed to different organs, including the central nervous system (CNS) through the blood. Upregulation of proinflammatory cytokines and diffuse activation of microglia locally in the neonatal brain also occur, releasing inflammatory markers, which could enhance brain injury (109, 111, 112). Activation of IL-1β produced by microglial cells in hypoxic conditions can delay the white matter development and recovery in hypoxic conditions (113, 114). Markus et al found an increase in cerebral gene expression of IL-1β after ventilation with 100% oxygen compared to room air after fetal asphyxia in a newborn sheep model.

It has also been reported that inflammatory markers such as tumor necrosis factor (TNF)-α, IL-1β, IL-2, -6, -8 and -18 enhance damage to the developing brain through different pathways, leading to periventricular white matter damage in the neonate (115, 116).

Matrix metalloproteinases (MMPs) play a significant role in brain damage and repair after hypoxia-reoxygenation. MMPs regulate tissue inflammation in response to oxidative stress (117, 118). MMP-9 has been shown to induce neuronal death (119-121), and MMP-2 has been found to play a role in neuronal damage (122, 123). In one observational study by Graham et al. (124) involving 126 patients with acute stroke, MMP-9 mRNA concentration was found to be almost three times higher in patients who died within 6 months, compared with patients who survived ($p=0.0002$). Caspase-3 is another marker that plays an important role in cell death. It is activated after hypoxia in the cerebral cortex (125).

In some studies, serum IL-1β and IL-6; and cerebrospinal fluid (CSF) IL-1β (all measured before 96 hours of age) were possible markers of abnormal outcomes at age ≥12 months in survivors (96). In one study of term newborns with HIE that were treated with hypothermia, levels of serum S100 correlated with neurologic outcomes (97).

Others have presented results, in accordance with this thesis, that confirm the association between inflammatory processes, brain injury and neurological findings in term newborn infants with HIE with elevated cerebrospinal fluid IL-6 and TNF-α levels (112, 126). Shalak et al. (127) also reported a significant association between abnormalities in the neurological examination and cytokine concentrations, with the highest cytokine concentrations (IL-6, IL-8) in term infants who developed clinical encephalopathy with seizures.

During the last twenty years, the protein S100B has received increasing attention as a possible biomarker for neurological disease (128, 129). Low serum levels of the protein are found in healthy individuals while patients with head trauma have a level of S100B proportionate with the severity of their brain injury (130). Serum S100B can detect early brain injury (131, 132) and change in response to secondary brain insults (133). Inflammatory mediators released acutely after ischemia-reperfusion by the blood-bain barrier`s epithelial layer may provoke astrocytes to release cytokines and S100B (134, 135).

In one recent study by Aridas et al they found that S100B concentration in CSF collected at 72 h after asphyxia, was elevated 5-fold in asphyxia lambs compared to control lambs (p=0.01), likely reflecting a CNS response to injury (136). In response to asphyxia, the source of increased S100B in CSF is likely to astrocytes (137). S100B has been examined in newborns with and without asphyxia and serum levels were associated with moderate-severe HIE from birth to 24 h, but not with neurodevelopment assessment at 20 months of age (138, 139). A discrepancy in the predictive utility of S100B may possibly reflect the balance between its neurotrophic and neurotoxic roles, and extracerebral resources such as adipocytes and bone marrow that may contribute to an injury response (137).

Another study by Mortberg et al. has found that S100B levels at 24h after ROSC (and treated with therapeutic hypothermia) predicted poor outcome with a specificity of 100% and a sensitivity of 87%. Outcome was assessed at discharge and at 6 months after resuscitation (140).

S100B has a very high sensitivity for brain injuries, possibly even higher than CT (141). It is therefore a promising screening tool that may be of help to support the clinician`s decision not to perform CT imaging in certain cases of low-risk head injury. In the Scandinavian guidelines for the acute management of adult patients with minimal, mild, or moderate head injuries, published in 2013, they now recommend a blood test of S100B as an initial diagnostic measure for mild head injury patients with low risk. Of these patients, CT examination is only recommended for those who show a pathologically elevated S100B (142).

However, there are no established biomarkers for determining the extent of neonatal brain injury or predicting outcome in infants with HIE. Further studies are needed to determine if any of these markers truly could be useful for the early assessment of infants with HIE and hopefully indicate if therapy should be started.

Resuscitation of the newborn and lung inflammation

Parts of this thesis aimed to assess the influence of cardiac arrest on lung injuries and to investigate some of the possible markers of inflammation and the mechanisms leading to lung injury.

Multiple ventilation strategies for resuscitation have been attempted to reduce lung injury and improve respiratory disease outcomes in newborn infants (143, 144). Ogden et al reported already in 1983 that large numbers of inflammatory cells were present in tracheal aspirates of infants that developed bronchopulmonary dysplasia (BPD), irrespective of lower respiratory infection or septicemia (145).

BPD is the commonest chronic lung disease of infancy (146), usually diagnosed in preterm infants at least 28 days after birth. Early recognition of infants at high risk of developing BPD would be critical to plan out therapeutical strategies. The inflammatory process is dependent upon the effective release and balance of cytokines. An imbalance in these mediators leads to activation of the cellular death pathways in the lung (147). The identification of these infants needs to occur within the first 3 days of the early phase of BPD for potential maximum impact (148). A variety of biomarkers have been proposed for early identification of BPD. MMP-9/tissue inhibitor of metalloproteinase-1 ratios in cord blood were in one study significantly higher in a study with infants who developed moderate/severe BPD versus those who had mild/no BPD (149). Some of the most promising biomarkers associated with BPD are, according to Bhandari et al, IL-1β, IL-6,
MCP-1, TGFβ1, VEGF, MMP-9 and IFNγ (146). Another review article found that promising biomarkers could be IL-10 and MMP-9 (150).

In 2009, Solberg et al found that resuscitation of hypoxic newborn pigs with supplementary oxygen induces a dose-dependent increase in activity of MMP-2 and MMP-9 in liver tissue (151). The increase indicates that using 40% or 100% oxygen when resuscitating newborn can cause tissue damage and influence remodeling processes. MMPs are important in ischemia-reperfusion injury where increased MMP-2 and MMP-9 levels are detected in multiple organs including the brain (152). Newborn pigs exposed to hypoxia have increased MMP levels in lung and liver as early as 2-2.5 h after reoxygenation with 100% oxygen (153, 154). MMP-9

MMP-9 is prominently expressed in the lung, and while it is involved in many normal homeostatic processes, dysregulated MMP-9 activity has been described in a number of lung diseases. Kong et al. found that MMP-9 is an important

biomarker of disease severity in mechanically ventilated children with RSV lung infection (155).
Lista et al. (156) evaluated the lung inflammatory response in preterm infants with Respiratory Distress Syndrome (RDS), mechanically ventilated with or without volume guaranty, by measuring pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) in tracheobronchial aspirate fluid. Their data suggest that a volume-targeted ventilatory strategy may play a role in reducing the acute inflammatory response, and thereby also limiting oxidative stress in preterm infants with RDS.

D'Angio et al speculated that lung injuries caused by asphyxia were mainly attributed to alveolar wall damage and depletion of surfactant proteins in a rabbit model (157).

Soluble intercellular adhesion molecule (sICAM-1) is a biological marker of alveolar epithelial and lung endothelial injury. Flori et al (158) examined plasma levels of sICAM-1 in pediatric patients with acute lung injury. Plasma sICAM-1 levels were significantly higher in patients with acute lung injury, which may also be the result of extreme hypoxia-reoxygenetion injury, compared with controls. Levels of sICAM-1 were also significantly higher on days 1 and 2 of acute lung injury in nonsurvivors and in patients requiring prolonged duration of mechanical ventilation. Early elevation of plasma sICAM-1 in pediatric patients with acute lung injury might therefore be associated with increased risk of death or prolonged duration of mechanical ventilation.

Cytokine responses during mechanical ventilation have been examined by Kobr et al. (159) in newborn pigs. They were intubated and divided into groups of A, spontaneously breathing; B, protectively ventilated; C, ventilated with injurious strategy; D, ventilated with lung disability. At the 1st hour (time-1) and 12th hour (time-2) of the study, brain natriuretic peptide (BNP), ICAM-1, TNF-α, and IL-6 were analyzed in blood. The injurious ventilated group C exhibited an increase in ICAM-1 and TNF-α at time-1, and at time-2 their levels further increased. The lung damage correlated with TNF-α, IL-6 and ICAM-1 levels. These studies are all in accordance with papers presented in this thesis. However, as pilot studies in our piglet asphyxia model indicated that the protein expression of several of the inflammatory markers of interest may be delayed beyond our 4 h observation period, we decided on also doing gene expression analyses in papers II and III.

Aims of the thesis

The overall aim with this thesis was to compare alternative resuscitation protocols with the accepted algorithm. Specifically, we wanted to investigate the following issues:

1) What is the optimal time of ventilation before initiation of cardiac compressions during resuscitation of the newborn? We hypothesized that longer ventilation intervals would result in a shorter time to ROSC (Paper I).

2) How would a longer period of ventilation before initiating cardiac compressions in newborns affect markers of inflammation? We hypothesized that a longer period of ventilation before initiation of cardiac compressions would result in a lower inflammatory response in the brain and lung tissue (Paper II and III).

3) What effect would alternative compression to ventilation ratios of 9:3 and 15:2 have compared to the standard 3:1 regarding inflammatory response? (Paper II and III)

4) We also wanted to measure the inflammatory response in the lungs and the brain when using air instead of 100% oxygen in severely asphyxiated pigs resuscitated from asystole (Paper III).

We chose the newborn pig model to investigate these aspects because it resembles humans in both anatomy and physiology of the cardiovascular system (7-9).

Methods and experimental models

The newborn pig model

We chose healthy newborn Noroc pigs for our experiments. Pigs have become a major translational research model over the last three decades (7). The similarities between the newborn pig and the human newborn make pigs an attractive experimental model for investigating the effects of hypoxia and ischemia in different organs. The comparative anatomy and physiology have been detailed and published (7, 8, 160, 161). The experiments underlying my thesis were performed in a pig model of a newborn at term. Our findings do not apply to other gestational ages.

The level of development of the neonatal pig´s brain is comparable with that of a term human newborn (162). The pig brain has the same amount of gray and white matter as humans and the physiology of the brain is similar in both species (163). Maximum neuronal growth and proliferation at birth in pigs are also similar to humans: the CNS in both species grows most rapidly 5-6 weeks before term until 5-6 weeks after birth (163, 164).

The newborn pig displays a very similar type of organ injury and cardiovascular response to global hypoxia-ischemia as in the human organs: It has a similar cerebral blood flow response to hypoxia-ischemia (165), similar vulnerability of specific cerebral regions (166) and similar pattern and time course of delayed energy failure (167). The circulatory response to changing levels of carbon dioxide and blood pressure is also quite similar to the newborn infant (168).

A limitation to our model is that adaption to extrauterine life has already begun when pigs are subjected to global hypoxia at the age of 12-36 hours. This is of particular importance with regards to investigating ventilation strategies, as fluid-filled lungs immediately after birth certainly require a different approach than fully aerated lungs as presented in our experimental animals. Further, although the breeder selected healthy pigs in good condition, there is a large inter-individual variability, especially in vulnerability to hypoxia. This increases the variation in the data, which further increases the numbers of animals necessary to detect effects of interventions. Also, the low hemoglobin frequently seen in domestic pigs make their susceptibility to hypoxia/asphyxia and resuscitation not entirely representative of newborn infants that generally have a relative polycythemia. However, the pig model has many advantages and is generally considered to be appropriate for neonatal CPR.

The experiments were performed in accordance with The Norwegian Animal Welfare Authority Act and were approved by The Norwegian Animal Research Authority. The use of a relatively large experimental animal such as the newborn pig allowed us to use the same equipment commonly used to treat human newborn infants in the delivery room and in the neonatal intensive care unit.

We modified the newborn asphyxia pig model developed through years at the Department of Pediatric Research, Oslo University Hospital, in collaboration with the Institute of Surgical Research, Rikshospitalet and University of Texas Southwestern Medical Center at Dallas, Texas, to a modified model where we induced cardiac arrest in the newborn pigs. Clinical and experimental studies to date have mostly studied individuals with mild or moderate asphyxia. We therefore wanted to investigate whether air could be used also in severely compromised newborn pigs with cardiac arrest.

The pigs were transported from a local farm on the day of the experiment and placed in a warm transport incubator to avoid hypothermia. Animals with hemoglobin concentrations lower than 6 g/dl or poor general condition (e.g. hypotension, bradycardia) were excluded.

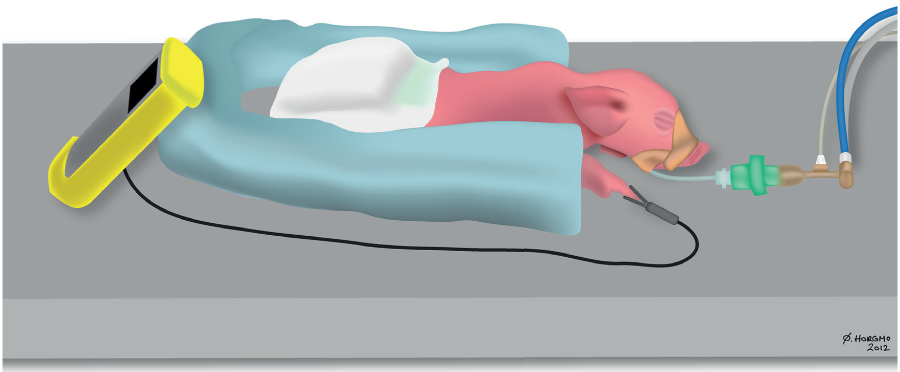

Figure 4. Illustration of our pig model made by Øystein H.Horgmo, Medical Photography Section, University of Oslo.

Asystole was defined as a MABP of 0 mm Hg with loss of pulsatility. Asphyxia was maintained and the ventilator rate reduced by 10 breaths per minute every 10 minutes until the heart rate was zero as judged by ECG (Electrocardiogram), invasive pressure tracings and cardiac auscultation. The ECG leads were placed behind the left and right ear and in the perineum, respectively, and ECG was continuously registered through the Biopac 150 and saved to a computer hard disk using the Acqknowledge® software (Biopac systems, Goleta, CA, USA).. The ECG tracings, unfortunately turned out quite coarse, and could not be used for detailed rhythm analysis, so some kind of electrical activity at a clinical heart rate of zero (asystole) cannot be entirely ruled out, buth then if this was the case, the animals might have been more easily resuscitated.

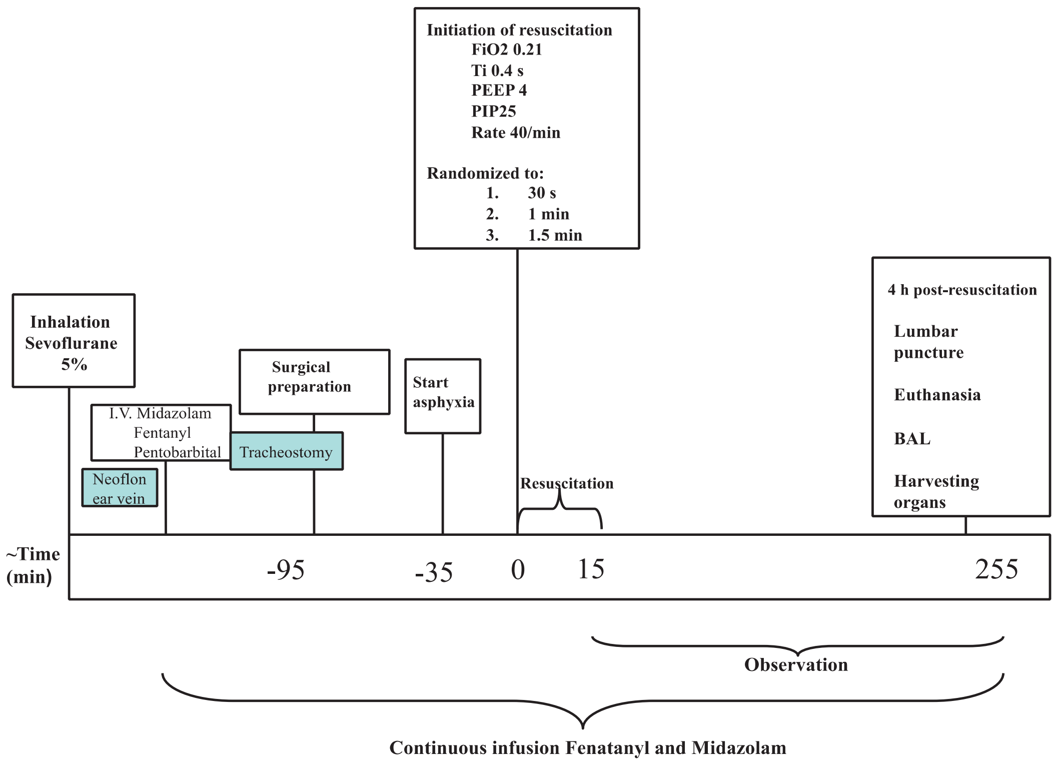

Figure 5. Experimental set-up for paper 1, 2 and 3 (169). Figure from paper I.

A limitation to performing resuscitation studies in this model is that sometimes ventilations and compressions inevitably would coincide which was not intended in this thesis. The question whether continuous and asynchronuous ventilations and cardiac compressions would be better or as effective as the 3:1 algorithm, was however raised at the 3rd Neonatal Resuscitation Research Workshop in association with the American Academy of Pediatrics in Washington 2012. After this meeting, studies have been performed by our research group (results in manuscript) and by others (170) exploring if the continuous and uncoordinated ventilation and compression approach would be easier to perform and further to find out if uncoordinated compressions and ventilations provide adequate tidal and minute volumes. This strategy does not seem to hinder the effectiveness of the chest compressions, as can be seen from arterial pressure tracings showing that inflations do not affect the pressures substantially (169, 171, 172).

We ventilated the pigs in need of resuscitation with the mechanical ventilator (Babylog) instead of using a resuscitation bag or T-piece device. In delivery room resuscitation, a resuscitation bag or a T-piece is routinely used for ventilation of the newborn. We used the mechanical ventilator for this purpose because this would give us more standardised ventilations. The ventilation was pressure controlled not volume controlled which is the way we ventilate newborn infants. Also, because of a limited number of hands, we found the use of the ventilator a feasible way of also managing cardiac compressions, adrenaline administration and coordination of the events.

Chest compressions were aimed to generate a clinically relevant arterial pressure. We aimed at a MABP of >20 mmHg and anticipated that ROSC depended on the effectiveness of the chest compressions. We administered adrenaline as per ILCOR guidelines (173), with the first dose (0.02 mg/kg) being administered after 30 s of cardiac compressions without signs of improvement in HR, and found that the vast majority of the experimental animals received ROSC only after adrenaline injections, in recommended doses and intervals, pointing to the need for this drug to obtain ROSC in newborn resuscitation. We questioned whether guidelines should advise adrenaline to be given earlier when the newborn is at cardiac arrest. However, in a later publication by Linner et al in asphyxiated newborn pigs, early adrenaline administration did not affect the time to ROSC (174). The blood pressure was continually monitored, recorded and saved.

Anesthesia

The studies were performed under general anesthesia. Dose levels of anesthetics for research animals have been developed through veterinary practice and in human medicine. Complete reviews of porcine anesthetic protocols, their physiologic effects, and indications have been published (160, 161). The combination of drugs used in our experiments has been developed through the years at the Department for Surgical Research at Oslo University Hospital, Rikshospitalet.

As an introductory anesthesia, the experimental animals were given Sevoflurane 2-5% (Sevorane, Abbott) before an ear vein was cannulated. The Sevoflurane gas was then disconnected as bolus intraveneous injections of Pentobarbital (15–20 mg/kg), Fentanyl (50 µg/kg) and Midazolam (0.5 mg/kg) were given and anaesthesia maintained by a continuous infusion of Fentanyl (50 µg/kg/h) and Midazolam (0.25 mg/kg/h). If necessary, a bolus of 50 µg/kg Fentanyl, 2.5 mg/kg Midazolam or 2.5 mg/kg Pentobarbital was administered according to clinical judgment. Pentobarbital was only added when there was increased muscular tone that did not respond to Fentanyl or Midazolam. Animals were euthanized by administration of 150 mg/kg Pentobarbital i.v.

Sevoflurane has been found to be a safe and effective choice of anesthetics for infants (175) and has also been used in pigs and other animal models both by veterinarians and in research (176, 177).

Pentobarbital is widely used in animal research and is known to have dose-dependent cardiovascular depressant effects (178). Animal studies have also shown a neuroprotective effect of barbiturates against hypoxic-ischemic brain damage (179-181). The doses in our studies were small and are unlikely to have influenced the results considerably.

Fentanyl was used for analgesia. According to Swindle et al (8), pigs have shown to require larger concentrations of opiates than humans and many other animals used for research. Fentanyl can cause a moderate reduction in cerebral blood flow and cerebral fractional oxygen extraction (FTOE) (182), but stable hemodynamic conditions have been found in pigs during continuous fentanyl infusion (183).

Continous infusion of Midazolam has been shown to increase cerebral FTOE in a newborn pig model, possibly through compromised cerebral perfusion and oxygenation (150). However, midazolam is an effective swine sedative that is associated with stable cardiac function (184).

Mechanical ventilation

The pigs were tracheotomized and mechanically ventilated with 21% oxygen, inspiratory time of 0.4 s, PIP 25 cm H_2O, positive end peek expiratory pressure (PEEP) 5 cm H_2O and rate depending on pCO_2 values (target range 4.5–6.0 kPa).

The piglets had normal lungs, but were ventilated with pressures that most likely would have contributed to over-ventilation of a healthy human neonate. Through years of experience with this porcine model and experimental setup with the ventilator, we believe that the porcine lungs are less compliant than those of humans, and a PIP of 25 cm H_2O and postive end expiratory pressure (PEEP) of 5 cm H_2O have been chosen after years of experience with these pigs in the department. The pCO_2 measured throughout the experiment, including the one hour stabilisation period indicates normoventilation with pCO_2 being > 4.5 kPa (data in paper I).

The studies described in this thesis were preceeded by a pilot study where we initially induced cardiac arrest by clamping the endotracheal tube. Surprisingly, these animals had a low pCO_2 at asystole, and we were not able to resuscitate them. We therefore started to hypoventilate them by stepwise reduction of the ventilator rate, and at the same time adding CO_2 to the inspiratory gases. A limitation may be that we did not test whether adding CO_2 really was necessary in order to achieve asphyxial levels of CO_2 in the hypoventilation model. An experimental model which produces higher pCO_2 levels would be more similar to asphyxia in newborns, and the metabolic component of acidosis would have been higher, ie higher lactate levels.

After our experiments were finished, Linner et al. published a paper where asphyxiated newborn pigs were disconnected from the ventilator after 20 min of hypoventilation and achieved a pCO_2 of about 20 kPa (185).

Central arterial and venous lines

The left external jugular vein was cannulated with an arterial cannula and sutured. The right common carotid artery was cannulated using a venflon-catheter for continuous blood pressure and Heart Rate (HR) monitoring. After the surgical procedure all animals were stabilized on the ventilator for 1 h before asphyxiation. Following the resuscitation protocol, the animals recovered for 4 h while anesthetized. When considered necessary due to pain and shivering, a bolus dose of Fentanyl or Midazolam was added as previously described. Arterial specimens (0.2 ml) for temperature corrected blood gas analysis, glucose and lactate measurements were drawn from the catheter in the common carotid artery immediately following surgical instrumentation (i.e. at the beginning of the 60 min stabilisation period), after 20 min, 40 min and 60 min (i.e. at the end of the stabilisation period, defined as “baseline”).

Arterial blood gases were also analysed every 5 min throughout asphyxiation and each of the first five minutes following ROSC. The blood gas data and a discussion of these are presented in paper I. The “at asystole” blood gases were from the last blood gas obtained before the heart rate reached zero, i.e. a maximum of 5 minutes before actual asystole. Blood gas data presented as “immediately after/following ROSC” was from the samples taken 1 min after ROSC.

Near Infrared Spectroscopy

Near-infrared spectroscopy (NIRS) is a non-invasive technology that enables estimation of the tissue oxygenation. NIRS uses the relative transparency of human tissue to light in the near-infrared region of the spectrum. NIRS is based on the same principles as the widely used pulse oximetry, but where pulse oximetry uses the pulsating signal and thereby selectively measures arterial blood, NIRS measures the light attenuation of the tissue as a whole and estimates oxygen haemoglobin saturation of the blood in all types of vessels. This means that venous blood contributes more to the attenuation than arterial blood simply because venous blood has a greater volume. The ratio of venous:arterial contribution is generally considered to be 75:25, although this has been found to differ between and within infants (186-188).
It is thus not surprising that cerebral tissue oxygenation in our experiments showed only a fair correlation with oxygen saturation measured by orinary oximetry. There is no ‘reference standard’ for tissue oximetry and different NIRS devices differ in absolute values (189).

A pediatric cerebral tissue oximetry probe was placed on the left side of the head in half of the pigs. We were not able to obtain NIRS-values for the other half of the pigs as we obtained NIRS-values only from those experimental pigs that were placed on the bench in the operating room where the cerebral oximeter was installed. Since randomisation to intervention was made after stabilisation for all animals, systematic allocation to NIRS-registration within each group of interventions could not be made. Regional cerebral oxygen saturation ($rScO_2$) was continuously recorded (with registrations every 4 sec) on an external computer hard disk. After the experiments, cerebral fractional oxygen extraction (cFTOE) was calculated using the formula (SpO_2-$rScO_2$)/SpO_2.

Randomization to experimental groups 1-7 in series 1 and 2

The studies underlying papers I, II and III are based on the same two experimental series, with randomisation to groups

Experimental series 1 to groups 1-5

1. 21% oxygen, 30 seconds of initial ventilation, compression:ventilation ratio 3:1 (the reference group)
2. 100% oxygen, 30 seconds of initial ventilation, compression:ventilation ratio 3:1
3. 21% oxygen, 60 seconds of initial ventilation, compression:ventilation ratio 3:1
4. 21% oxygen, 90 seconds of initial ventilation, compression:ventilation ratio 3:1
5. 21% oxygen, 30 seconds of initial ventilation, compression:ventilation ratio 9:3

Randomization was made by pulling folded cards from a bowl, and stratification was made with respect to gender. In the following analysis of the results, group number 1 served as reference group for comparison against the other groups.

The power calculations we did for series 1 was based on pilot experiments comparing the effect of 21% and 100% oxygen in this newborn pig model of neonatal asphyxia and cardiac arrest. In order to detect a difference of 50 S in time to ROSC between treatment arms with a power of 80% and a significance level of 5%, the number of pigs needed (n) in each study arm (group) was 16. However, importantly, the sample size pertained only to the outcome of ROSC. Separate power calculations were not made for other outcomes, including inflammatory markers.

Results in the experimental series 2 with groups 6 and 7 is based on experiments performed about one year later, with 11 animals in each group, and after a renewed power estimate based on the ROSC-data from the pigs resuscitated with 100% oxygen and air (C:V ratio 3:1, initial ventilation for 30 seconds) in the series 1. In series 2, randomization to intervention (C:V ratio 15:2) or the reference group (C:V ratio 3:1), both in 21% oxygen, was made by drawing cards from a bowl, and stratification was also in this series made with respect to gender.

Experimental series 2 to groups 6 and 7:

6. 21% oxygen, 30 seconds of initial ventilation, compression: ventilation ratio 3:1 (the reference group)
7. 21% oxygen, 30 seconds of initial ventilation, compression: ventilation ratio 15:2

For power analyses, in both series a difference of 50 seconds between the groups was used for the time from asystole to ROSC. We had no comparable outcome data, so we had to determine what would be a ”clinically significant difference” in our studies. Based on preliminary pilot data, we set this difference to be 50 seconds. However, what really is a clinically relevant difference in ROSC in this model is still not known.

Cerebrospinal fluid and bronchoalveolar lavage fluid

Immediately before euthanasia of the animals, cerebrospinal fluid (CSF) was collected through a lumbar puncture, quite similar to the technique used in human newborns, using a 23 G needle. Immediately after death, bronchoalveolar lavage was perfomed and bronchoalveolar lavage fluid (BALF) collected using 30 ml/kg saline at temperature 37ºC. Aspirated BALF samples were immediately centrifuged at 2000×g and 4 ºC for 20 min to remove cells. The supernatant was transferred to polypropylene tubes (Nunc vials) and frozen at -80 ºC for later cytokine analysis. 0.5-1 ml of CSF was preserved for analysis of lactate and pyruvate, in addition to cytokine analyses. The lumbar punctures were done according to the same principles, procedures and with equipments as in human newborns when this is indicated.

Methods for quantification of inflammatory markers

The acute response protein S100, cytokines IL-6, IL-8 and TNF-α, adhesions molecule ICAM-1, apoptose markers caspase 3 and metalloproteinases MMP-2 and MMP-9 were analysed in CSF and tissue specimens from the brain (paper II) and/or in bronchoalveolar lavage and lung tissue (paper III). Methods were:

i) Enzyme-linked Immunosorbent Assay (ELISA) for S100 and the cytokines, a Spike and Recovery Immunoassay and Sample Validation Protocol from R&D systems, Inc (Minneapolis, USA) which is explained in papers II and III. CSF and BALF samples were analyzed in singlets with a twofold increase in volume of standard, control and sample added on R&D systems Quantikine for Porcine cytokines. Alternative quantitative techniques and measures could have been used like SDS gel electrophoresis and immunoblotting techniques but these are more time consuming and less accurate, and not so well established in our laboratory at the Epigen Institute at Akershus University Hospital.

ii) The Reverse Transcription-Quantitative Real-time PCR (RT-qPCR) of the tissue specimens were performed for genes IL-6, ICAM-1 TNF-α, MMP-2 and MMP-9, and caspase 3 after homogenization of tissue samples as described in papers II and III. The quantity and quality of total RNA was assessed using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). The High Capacity cDNA Reverse Transcription Kit (Part Number 4368814, Applied Biosystems, USA) was used for reverse transcription into complementary DNA (cDNA). The plate was run on the ABI PRISM 7300 Real-time PCR System (Applied Biosystems) with universal instrument settings. ABI Prism SDS1.3.1 software (Applied Biosystems). Acceptable NTC values were obtained. Peptidylprolyl isomerise A (PPIA) was used as a reference gene for normalization. Relative expression was determined by the comparative C_T method of relative quantification (RQ), calculated with the arithmetic formula $2^{-\Delta Ct}$, where ΔCt is the normalized signal level in a sample (ΔCt = Ct of target gene – Ct of reference gene).

The choice of inflammatory markers

Results from clinical and experimental studies are somewhat conflicting regarding cytokines that are up- or downregulated in perinatal asphyxia and at what time. There is probably an interplay in the network and pathways between several inflammatory and anti-inflammatory factors that are regulated by other factors. In experimental animal studies, a huge range of models of perinatal asphyxia, as well as differences in tissue- and fluid preparation, make comparisons between the different studies difficult. It was therefore challenging to find which cytokines and inflammatory markers which were likely to be involved in the inflammatory processes associated with brain and lung injury as a consequence of asphyxia. Likewise it was not obvious at what time frame specimens should be collected. We therefore tested methods for cytokine analysis of tissue homogenates and fluids, CSF, BALF and plasma. Protein concentrations in plasma were generally undetectable with porcine ELISA kits, as were proteins in tissue homogenates. These control animals had been manipulated with instrumentation (intubation and placement of venous and arterial catheters), but had not been asphyxiated and resuscitated, only euthaniazed after 4 h corresponding to the other animal's postasphyxiation time. Fully aware of the uncertainty of the role of the chosen markers in asphyxia and the time-course of their expression, we decided to use the presented inflammatory markers (papers II and III) in these studies.

The timeline of the experiments and analyses of inflammatory markers

We chose four hours after asphyxia as an appropriate time to expect changes in the set of cytokines we chose to analyse. These cytokines and inflammatory markers have somewhat different time courses, and are maximally expressed at varying time points. The cytokines´ maximal expression time point may also vary with the age of the pig and may be different from that of a human being. We performed a preliminary study prior to the experimental studies in this thesis that showed an increase in IL-1β in the brain and lungs of asphyxiated animals 4 hours after asphyxiation. Neonatal animal data also indicate that the expression of mRNA and bioactive proteins in brain extracts for the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α increase 1-4 hours after hypoxia-ischaemia (190, 191). This made us decide on measuring the inflammatory markers in brain and lung 4 hours after asphyxia. As tissue homogenisation gave undetectable levels of cytokines, we decided to analyse only CSF and BALF, as representatives for the brain and lung tissue, respectively, for quantification of inflammatory proteins, possibly reflecting expression of these markers in the organs of interest.

Clinical markers

The clinical characteristics at baseline in the two reference groups of series 1 and 2, respectively, are presented and compared in table 2 as mean (SD) of HR and MABP, showing that the groups have similar hemodynamic parameters, but differed in weight and lactate concentration at baseline. The baseline data, i.e. MABP, HR, in addition to blood gas values were from the end of the stabilization period. All data, inflammatory markers and clinical data, were compared to the reference group in each series; groups 2-5 compared to group 1, and group 7 compared to group 6, and differences between series 1 and 2 animals in weight and lactate levels should not influence on results and conclusions.

	Group 1, series 1, *n* = 16	Group 6, series 2, *n* = 11	*P* value
Age (h)	26 (6)	30 (8)	0.16
Weight (g)	2,270 (152)	2,531 (160)	<0.001*
Male/female	8/7	6/5	1.00
HR (bpm)	151 (21)	167 (37)	0.17
MAP (mm Hg)	60 (14)	60 (17)	1.00
pH	7.38 (0.07)	7.30 (0.1)	0.02*
BE (mmol/l)	−1.7 (3.6)	−1.7 (2.9)	1.00
pCO2 (kPa)	5.5 (1.4)	6.5 (1.1)	0.06
SpO2 (%)	90 (6)	88 (13)	0.60
Glucose (mmol/l)	7.8 (1.9)	7.9 (1.9)	0.90
Lactate (mmol/l)	2.9 (0.9)	1.3 (0.5)	<0.001*
Arterial Hb (g/dl)	7.1 (0.9)	7.1 (0.8)	1.00

Table 2. Clinical characteristics at baseline in the two reference groups. Table from paper II (169). The ultimate primary outcome in these experiments was time to return of spontaneous circulation. *P<0.05 between reference groups.

Statistical analysis

The choice of statistical method depends on the plausibility of normal assumptions, the importance of obtaining a confidence interval and the ease of calculation.

The probability that a test will produce asignificant difference at a given significance level is called the power of the test. This will depend on the true difference between the populations compared, the sample size and the significance level chosen. We have greater power if the p value chosen to be considered as significant is larger.

In paper I the descriptive statistics are reported as mean and standard deviation (SD) or median and interquartile range (IQR) for symmetrical and non-symmetrical parameters, respectively. To compare groups we used Student's t tests for symmetrical variables and Mann-Whitney tests otherwise. For categorical variables we used Fisher's exact test. A Bonferroni correction was used for multiple comparisons in paper I. P-values < 0.017 were considered statistically significant. To adjust for potential confounding factors we used regression analyses. Number of animals in this study was based on ROSC data from a pilot experiment comparing 21% and 100% oxygen in the same animal model. We performed a power analysis to detect a difference in 50 s in time to ROSC between the groups with a type I error rate of 0.05. Statistical analysis was performed using SPSS 14.0 for Windows (SPSS Inc., Chicago, Ill., USA).

In paper II we used two independent sample t-tests for symmetrical variables for comparison between groups. Otherwise, the Mann–Whitney test was used. Descriptive statistics were presented as mean (SD) or median (IQR). P-values <0.05 were considered statistically significant. P-values were not Bonferroni-corrected. Power analysis was performed as described under paper I. Statistical analysis was performed using SPSS 15.0 for Windows (SPSS, Chicago, IL)

In paper III we used two independent samples t-tests for symmetrical variables for comparison between groups. Otherwise the Mann-Whitney test was used. P-values were not Bonferroni-corrected. A power analysis was performed as described under paper I, leading to 16 animals in each group. Statistical analysis was performed using SPSS for Windows (SPSS Inc., Chicago, Ill., USA), version 15.0. The GraphPad Prism program (version 5.0c) was used to make graphical presentations.

Discussion

The experimental model

Based on the three papers included in this thesis, regarding both clinical injury and inflammatory response results, one may conclude that it is beneficial to initially ventilate a newborn for a longer period than the period of 30 s recommended by guidelines for neonatal resuscitation. Initial ventilation before compressing the chest to reestablish perfusion and circulation to body organs should, according to results in this thesis, last for a period between 60 s and 90 s, but an initial ventilation period of 90 s seems too long.

When we planned and initiated these pig experimental series, the resuscitation algorithm for the initial ventilation time was 30 s before cardiac compressions should be started, if persistent bradycardia below 60 beats per minute. During the past few years both alternative ratios of C:V and the ventilation time before onset of cardiac compressions, have been debated at several newborn resuscitation seminars and workshops. The papers in this thesis are to our knowledge the first to suggest that ventilation for 30 s may be too short before chest compressions should start. After our experimental series, others have reported on cardiac compressions during inflations (192), as well as uncoordinated cardiac compressions with ventilations (170) or alternative ventilation ratios (193).

The unique physiology of the newborn with initially fluid-filled alveoli, the need to transition from fetal to newborn circulation, the open ductus arteriosus, and the frequent presence of severe asphyxia as the cause of cardiovascular collapse, adds dimensions that are difficult to test in animal models. But experiments on human newborns are difficult to manage and and in some instances unethical, especially when we are dealing with issues of cardiac arrest. We chose to do our experiments in a model that was available for us in Norway, which was the newborn pig model. This pig model has several similarities to human newborns regarding cardiac and pulmonary anatomy, size, immunology and susceptibility to hypoxia (8, 9). The distribution of grey/white matter (163), coronary blood flow (194), growth of the cardiovascular system(7) and neonatal pulmonary development is also comparable with a term human newborn. It is therefore reasonable to believe that our findings primarily apply to term infants.

Regarding methodological considerations, this pig model has been in use as a well-established model of perinatal asphyxia for several years and has resulted in many PhD theses in the Department of Pediatric Research at Rikshospitalet. The advantages and disadvantages of this model have been considered thoroughly throughout the years. Even though this model traditionally has not taken the animals as far as cardiac arrest as was the case in this thesis, the advantages of experience and years of trying and failing were valued when we decided on using the one-day-old pigs.

A limitation to performing resuscitation studies in this model is that ventilations and compressions inevitably could coincide which was not intended in this thesis. The question if continuous and asynchronuous ventilations and cardiac compressions would be better or as effective as the 3:1 algorithm, is not raised here, but has after these experimental series been questioned by us (manuscripts in preparation) and others (170) are exploring if the continuous and uncoordinated ventilation and compression strategies would be easier to perform. Another question is if uncoordinated compressions and ventilations would hinder effective ventilation(s) with adequate tidal and minute volumes. According to our experiments this strategy does not seem to hinder the compression effectiveness, as can be seen from arterial pressure tracings showing that inflations do not affect pressures substantially (169, 171, 172).

A potential weakness of our study design is the low hemoglobin in our pigs (6.6-7.3 g/dl) that potentially could influence oxygenation and hemodynamics as discussed previously. Also, the experiments were performed in animals that had undergone transition from intra- to extrauterine life, which occurs very quickly after birth in the pig. A previous study by Odland et al has shown that these animals do not have hemodynamically relevant patent ductus arteriosus, making their hemodynamics different from newborns in a delivery room (195). Fugelseth et al. also showed in a study of asphyxiated newborn pigs that approximately 10% of initially closed ducts actually reopened during asphyxia (196). However, as stated by the AHA, the Neonatal Resuscitation Guidelines '... are also applicable to neonates who have completed perinatal transition and require resuscitation during the first few weeks to months following birth'(33).

ROSC was defined as a heart rate ≥ 100 beats per minute. We were able to catch the exact moment where the heart rate reached 100 per minute only in a minority of animals. Invasive pressure tracings, heart rate and blood pressure at ROSC rose within a few seconds to higher levels than levels measured before induction of asphyxia. Hence, the observed heart rate "at ROSC" was often higher than 100 beats per minute. The time it took for the heart rate to increase from approximately 60 per min to more than 100 beats per minute was fairly short and as dicussed above, quite impossible to define exactly.

Linner et al (185) noted the same in a similar pig study investigating time to ROSC after resuscitation with 21% or 100% oxygen: "Once effective heart action was being reestablished, HR increased very quickly in all three groups: from below 100 bpm to over 200 bpm within 15 s". In agreement with Linner and our experience with this model, a 15 s relative imprecision in defining the exact time to ROSC would not be of significance to the conclusions drawn from our studies as we defined that a clinical significant difference in time to ROSC would be 50 s.

The asphyxiated animals were considerably sicker with extreme acidosis than most asphyxiated newborns we see in the clinical setting. It may be wrong to resuscitate such very sick newborn babies in real life, even when hypothermia treatment is available. At a mean pH of 6.68 and mean base excess of –28.3 mmol/L, myocardial function was severely depressed. Despite this, we were able to achieve ROSC in all, except a few animals. Of particular interest is that all experiments were performed in 21% oxygen and the pigs obtained ROSC relatively easily.

The use of extra oxygen in resuscitation

When we initiated our study, guidelines for resuscitation of neonates had last been published in 2005 (74). ILCOR in 2005 did not specify the concentration of oxygen to be used at initiation of resuscitation. They did state that there was no evidence to support a change in the oxygen concentration initially given, once adequate ventilation was established, and they recommended that supplementary oxygen should be considered for infants with persistent central cyanosis (74).

We are, to our knowledge, the first to show that resuscitation of asystolic newly born pigs with with air is as effective as 100% oxygen at least when the arrest interval is brief (85). The most important finding is however that use of air during resuscitation in human newborns is associated with lower mortality. Systematic reviews and meta-analyses show that neonatal mortality can be reduced by 30% in newborns resuscitated with room air (68, 69).

Inclusion of animals and group size

Inclusion of experimental animals into group 4 (21% oxygen, 90 seconds of initial ventilation, C:V ratio 3:1) was stopped when 8 out of 16 pre-planned pigs had been included. We considered it unethical to continue enrolling pigs to this group, as the median ROSC time when half of these animals were included, was much longer than in all other experimental animals. One may argue that this may have biased the results and made us conclude wrongly with assumptions based on inadequate numbers of subjects.

Another limitation of the studies underlying this thesis related to the question of sample size, is the fact that we did not perform a Bonferroni adjustment of the significance level in paper II and paper III. When performing multiple comparisons, the risk of finding differences between interventions by chance increases. The rationale for not performing Bonferroni corrections was that we would let the numbers ‘speak for themselves’ and leave it to the reader to determine the clinical relevance of the results. This should have been expressed more clearly in the papers, and the conclusions drawn by us should have been more conservative. The potential for multiple molecular biological comparisons for hypothesis generation, not hypothesis testing should also have been discussed in the papers.

The use of animals in research in Norway is regulated by the Norwegian Animal Welfare Act. The so-called “Three R’s” (Refinement, Reduction and Replacement) have had a major impact on legislations and ethical guidelines concerning animal research worldwide ref. This means that in the case where the researcher does not find a feasible alternative to doing animal experiments (replacement), even though the researcher feels comfortable that the animals do not suffer more than necessary (refinement), the number of animals used for experiments should be kept to the absolute minimum required for the experiments (reduction).

These principles lead to the decision that we would conduct the experiments in a manner where several alternative approaches to resuscitation were compared to the same reference group. Also, the decision to stop inclusion to one of the groups (initial ventilation for 90s, group 4) because the time to ROSC always was about doubled of what we had experienced in the other groups. Our decision to stop inclusion in the 90 s initial ventilation group was guided by the principle of reduction. In this principle lies that reduction should be done without compromise of the scientific output and the quality of the research. Some might argue that the decision made by us in this case made our conclusions less reliable.

Discussion of results paper I

Delayed onset of cardiac compressions in cardiopulmonary resuscitation of newborn pigs with asphyctic cardiac arrest

This is a study on the optimal time of ventilation before initiating cardiac compressions in asphyxiated newborn pigs in need for intensive resuscitation. We could not prove that ventilation for 60 s before initiation of cardiac compressions led to a shorter or longer time to ROSC compared to the recommended 30 s of initial ventilation, but 90 s was too long since it led to significantly longer times to ROSC. Furthermore, those experimental animals that died were within this 90 s-initial-ventilation group

We did not find differences between groups of 30 s versus 60 s of initial ventilation in time to ROSC or hemodynamic parameter in the observational period of 4 h after ROSC. Neither did we in papers II and III find differences in the biomarkers of hypoxic injury or inflammation between these 2 groups. When delaying initiation of cardiac compressions to 90 s, time to ROSC was significantly longer and subsequent hemodynamic outcomes were markedly worsened. Our results seem to indicate that the optimal time with efficient ventilation may be longer than the currently recommended 30-60 s.

A weakness of this study may be that we decided to stop inclusion in the 90 s group when 8 animals were included. As these animals differed from the animals in the other groups for some baseline features (higher HR, lower pH and lactate), one might ask if this group was entirely comparable with the other 2 experimental groups. However, after discovering these differences, we calculated baseline measurements for the first 8 animals in each of the 3 groups. We saw then that the documented differences in the 90 s group were already present when the first 8 animals were included in every group; hence the differences were more likely due to chance than incomplete randomization. The differences do not appear to be clinically relevant since the variation is clinically small. It is also worthwhile noticing that when we compared times to ROSC halfway through the study, the difference between 30 s and 90 s was already significant ($p = 0.005$). Also, as group sizes of 8-10 animals are frequently being used in large animal models like pigs and sheep, this was another aspect that we took into account when the decision was made not to put more animals through a protocol that so apparently seemed to worsen the outcomes of the animals. Surely, this decision may have resulted in both performance, detection and reporting bias.

Still, we think there is reason to conclude that a longer ventilation period than the 2005 ILCOR guidelines for 30 s and the 2010 guidelines for 30-60 s should be evaluated with respect to these clinical results, and also with respect to inflammatory signs of brain (paper II) and lung injury (paper III). Also, with regards to the fact that compliance with the current guidelines has been shown to be poor (197) the short time allocated to ventilation before proceeding to chest compressions should continue to be questioned. Adequate ventilation performed long enough seems to be important.

According to laboratory characteristics immediately after resuscitation one of the conclusions in Paper I, is that pCO_2 at ROSC is significantly higher after 1 min of initial ventilation as compared to 30s of ventilation before initiation of cardiac compressions. The clinical relevance of higher pCO_2-values with prolonged intervals of initial ventilation (both in the 60s group and the 90s group) is uncertain, but might be subject to further studies. However, animal data indicate that higher pCO_2 following asphyxia restores cerebral microcirculation faster and that mild hypercapnia is associated with less severe brain injury following hypoxia-ischaemia (82). Moderately high pCO_2 might also reduce cellular oxygen demand and facilitate oxygen delivery (82).

Discussion of results paper II

Brain inflammation induced by severe asphyxia in pigs and the impact of alternative resuscitation strategies on the newborn central nervous system

In this study we compared current guidelines for neonatal resuscitation to alternative methods to determine if this would modulate inflammatory markers in the frontal cortex, hippocampus and CSF. In CSF, S100 was higher when the pigs received initial positive pressure ventilation of 90s as opposed to 30s or 60s. Concentrations of IL-6 and TNF-α in CSF were higher in the groups ventilated for 30s than 60s. We cannot fully explain this finding, but it may be due to the 30s longer period of chest compressions probably inducing more pulmonary and/or chest trauma in the pigs ventilated for 30s instead of 60s before onset of compressions. One may speculate that inflammatory markers produced systemically as a result of chest trauma may travel to the CNS through the blood stream and cause brain inflammation, which in turn possibly affects the neurological outcome. The association between inflammatory markers and HIE has been demonstrated (101). RT-PCR of MMP2 and ICAM-1 were also higher in the 30s group compared to the 60s group, which again could point to the fact that 60s should be preferred over 30s. We therefore concluded, with respect to brain injury, that a longer ventilation period than the recommended guidelines of 30s should be considered but 90s of initial ventilation without chest compressions seems too long. This conclusion should be interpreted in the light of the fact that our animals were asystolic, and that the theoretical rationale for the effectiveness of ventilating lungs that are not circulated is somewhat lacking. In retrospect, we realize that the research question probably would have been more appropriately answered in a bradycardia model. Also, as bradycardia is a more frequent cause for a need for cardiac compressions in the delivery room than asystole, the choice of model can and should be questioned. Still, with these limitations in mind, time to ROSC in this model was comparable in animals ventilated for 60 and 30 s before circulation was provided with cardiac compressions.

The alternative compression to ventilation ratios (C:V) of 9:3 and 15:2 compared to 3:1, or the use of air instead of pure oxygen in these extremely acidotic and sick pigs at asystole, did not change the inflammatory brain response. We could not prove any up or downregulation of inflammatory markers with alternative C:V ratios compared to the reference group, at least according to the inflammatory markers studied in this thesis.

One could argue that a time-response experiment for each inflammatory parameter should ideally have been performed. This would have necessitated many more experimental animals in order to find the optimal time point to study each of the inflammatory parameters. Then we could have collected samples at the optimal time point for each parameter. With a power estimate of 16 animals in each group for each parameter, this would have necessitated an immense number of animals. Therefore, instead we decided on a single 4 h post-ROSC observation time after the hypoxia-reoxygenation injury and reestablished ROSC, meaning that all inflammatory markers were analysed at the same time (4 h post-ROSC), whereas other laboratory and clinical parameters were measured throughout the experiment (Paper I).

We have no obvious explanation why an initial ventilation period of 60 s versus 30 s before initiation of chest compressions produces lower CSF concentrations of IL-6 and TNF-α and less expression of hippocampus MMP-2 and ICAM-1 genes. The differences are small between the groups, maybe except for S100 in CSF, so the biological significance may be questionable. One suggestion may be, as mentioned above, the longer period with chest compressions in the reference group leading to 40-50 more cardiac compressions, and therefore possibly more organ injury and then higher levels of inflammatory markers, including responses in the central nervous system. Although the inflammatory differences between the 30 s reference group and the 60 s group are tentative and small, as mentioned aerlier, we speculate that the chest trauma from cardiac compressions produces circulating pro-inflammatory mediators that can be measured in CSF. Small differences were also measured in the expressions of inflammatory genes in the brain tissue as is also reported in mouse models of blunt trauma (198). The longer ventilation interval meaning less chest compressions reduces the risk of extensive trauma with rib fractures and pulmonary contusion, which we observed (anectdotally, not measures as an outcome parameter) in some of our newborn pigs.

The laboratory characteristics (pH, BE, pCO_2) for 9:3 compared to 3:1 in experimental series 1, and for 15:2 versus 3:1 in series 2, immediately after ROSC, was not significantly different between the various C:V groups which is in accordance with inflammatory responses with ratios 9:3 and 15:2 compared to 3:1 in paper III.

Discussion of results paper III

Lung injury in asphyxiated newborn pigs resuscitated from cardiac arrest - the impact of supplementary oxygen and longer ventilation intervals before initiation of chest compressions at different compression-to-ventilation ratios.

In this study we focused on lung injury and investigated the levels of lung inflammatory markers by comparing the different ventilation, cardiac compression and inhaled oxygen fraction strategies in resuscitation of asystolic newly born pigs at cardiac arrest. BAL-levels of TNF-α and IL-8 tended to be higher in the 30 s initial ventilation group as compared to the 60s initial ventilation group as was gene expression in lung tissue of ICAM-1 and MMP-2. Altogether this points to 60 s as superior to 30 s of initial ventilation. MMP-2 expression also tended to be slightly higher in the 30 s group compared to 90 s group. Compared to current guidelines, with respect to lung injury, we believe that resuscitation with longer initial ventilation periods should be considered. Longer series of cardiac compressions (C:V of 15:2 and 9:3) did not change the the levels of inflammatory markers in BALF or lung tissue, neither did the use of air instead of pure oxygen in severely asphyxiated pigs resuscitated from asystole. Even though air ventilation has previously been shown to reduce inflammation compared to ventilation with 100% oxygen, the lack of difference in our model may be explained by the physical trauma component to inflammation being larger than the oxidative stress component.

The differences in inflammatory markers are small so we suggest that factors in addition to inflammation in tissues should be taken into account when guidelines are being revised. For instance, the ease of performing and teaching a strategy could potentially influence the clinical effectiveness of the strategy and should also be taken into account. The small inflammatory differences between 30 s, 60 s and 90 s of initial ventilation reflect that many factors influence the intensity of lung injury including the actual number of cardiac compressions during resuscitation, the severity of asphyxia, reoxygenation and the fraction of oxygen used during ventilation, as well as the time to return of ROSC. Based on the results in this paper, we again argue that a longer period with effective, initial ventilation should be considered as an alternative to current guidelines.

Ideally, to explore if there are any differences between various resuscitation strategies, a time-response experiment in lung tissue and BALF should have been performed for every inflammatory marker, in order to define the optimal time point for measuring the inflammatory response. We did not perform such time-response experiments and we cannot be sure if other time points should have been chosen and possibly could have shown differences between the different initial ventilation periods. This was due to animal ethical considerations, evaluation of cost-benefits and in accordance with Norwegian standards since it would have necessitated a large number of animals to perform time response experiments in both lung and brain tissue (paper II) for all inflammatory markers and then run a series for every parameter at its specific and optimal time point. We therefore reviewed the literature and decided on 4 hours post-resuscitation time to be the best general time-point for all parameters. The choice of which inflammatory markers to analyse to prove lung injury was also based on a literature research, i.e. MMPs are important in ischemia-reperfusion injury (117, 118, 123), and we had some experience in the research group analysing these factors (153). Further, as it is known that nonspecific inflammatory events following lung injury or severe asphyxia involve IL-8, TNF-α and ICAM-1 (156, 158, 159), these markers were included in the experimental panel.

Conclusions

1) In paper I we studied the optimal time of ventilation before initiating cardiac compressions in asphyxiated newborn pigs in need of resuscitation. There was no difference in time to ROSC between the animals resuscitated with an initial ventilation period of 30 s compared to 60 s. When delaying cardiac compressions with 90 s of initial ventilation, time to ROSC was significantly delayed.

2) 60 s of ventilation before initiating cardiac compressions during resuscitation instead of the current algorithm of 30 s might induce less lung and brain inflammation in newborn pigs (Paper II and III).

3) The compression to ventilation ratio of 9:3 and 15:2 seems to be as gentle as 3:1, according to the inflammatory and injury markers we measured in the newborn pig brain and lung tissue (Paper II and III).

4) We did not find differences in inflammatory responses when the severely asphyxiated pigs were resuscitated with air or 100% oxygen (Paper III).

Future perspectives

Intensive cardiopulmonary resuscitation involving cardiac compressions and medications is an infrequent event, and randomized controlled studies are not easily undertaken. Such studies are also problematic due to ethical considerations regarding the treatment of an acutely compromised newborn. An interesting and unanswered question by our pig studies is whether increasing the C:V ratio in the newborn compromises ventilation, or whether alternative C:V ratios or uncoordinated compressions and ventilations may be more appropriate in terms of tidal and/or minute volume delivery. Another interesting question is whether alternative strategies may be easier to perform and possibly be less exhausting which is one of the research questions focused in our research group at the moment. We are therefore planning to perform further studies on the optimal cardiac compression and ventilation strategies on a newborn manikin model. Also, the efficacy of various ventilation rates (presently recommended 40-60/min) can be studied in this model.

The 2010 ILCOR neonatal resuscitation guidelines do not specify the optimal inflation time for the initial lung inflations in apnoeic newborn infants. Several studies have shown that sustained inflations during resuscitation may improve functional residual capacity by allowing the air/liquid interface to move into the distal airway (42, 199). One recent study also found that a combination of chest compressions during sustained inflations could improve the return of spontaneous circulation with better hemodynamic recovery in asphyxiated newborn pigs in comparison with standard 3:1 resuscitation (192). Sustained inflations could be regarded as a promising intervention, but further randomized trials in term and preterm infants are needed.

Most newborn resuscitation research has focused on specific elements of ventilation support and the initial resuscitation. Despite these important efforts, poor patient outcomes still persist and only minor changes are made in the 5-yearly updated ILCOR treatment recommendations. The 2015 ILCOR worksheets indicate that no changes will be made with regards to C:V ratio or the use of supplementary oxygen during cardiac compressions. Perhaps an important take home message is that whatever ventilation duration or C:V ratio is used, good quality ventilation and cardiac compressions should be delivered. A more comprehensive resuscitation education emphasizing appropriate use of interventions could have a major positive impact on resuscitation outcomes. Together with anesthesiologists and collaborators at the Childrens Hospital in Philadelphia, our research group has developed a clinical newborn resuscitation training program, INSPIRE-D, which soon will be in use at the delivery units at Akershus and Oslo University hospitals.

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Errata

In paper III there is an error in the text under the result section ”Variations in ratios of chest compressions-to-ventilation” on article page 93: the TNFα value in BAL in the group 4 resuscitated with a C:V ratio of 9:3 is not 1.38 (0.86-1.81), it should be 1.26 (0.92-1.55).